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ORIGINAL ARTICLE

Dual targeting of PD-L1 and PD-L2 by PCED1B-AS1 via sponging hsa-miR-194-5p induces immunosuppression in hepatocellular carcinoma

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Abstract

Background PD-L1 and PD-L2 are PD-1 ligands (PD-Ls). PD-Ls over-expression is associated with poor prognosis in hepatocellular carcinoma (HCC). However, little is known about how PD-Ls expression is regulated. Here, we investigated the involvement of lncRNA-microRNA network in the regulation of PD-Ls in HCC.

Methods The expression of PD-Ls, PCED1B-AS1 and hsa-miR-194-5p was measured in 45 pairs of HCC samples. The interaction between PCED1B-AS1 and hsa-miR-194-5p was measured by microRNA pull down and in vitro binding assay. The effects of PCED1B-AS1 knockdown and over-expression on hsa-miR-194-5p and PD-Ls expression were investigated in HCC cell lines. Immunosuppression was evaluated in co-culture of HCC cell line and human T cells. Exosomes were isolated from HCC cells and their effects on receipt cells were investigated. Tumor behaviors were evaluated by in vitro and in vivo assays.

Results PD-L1 expression was highly correlated with PD-L2 expression in HCC. PCED1B-AS1 and hsa-miR-194-5p expression was up-regulated in HCC. PCED1B-AS1 was positively correlated with PD-Ls but negatively correlated hsa-miR-194-5p in HCC. These correlations were cross-validated by TCGA-LIHC dataset. PCED1B-AS1 interacted with hsa-mir-194-5p which inhibited PD-Ls expression. PCED1B-AS1 enhanced the expression of PD-Ls via sponging hsa-mir-194-5p. PCED1B-AS1-induced PD-Ls-mediated immunosuppression in co-cultured T cells. HCC cells released PCED1B-AS1 containing exosomes and the exosomal PCED1B-AS1 enhanced PD-Ls expression in receipt HCC cells while inhibited receipt T cells and macrophages. Blood exosomal PCED1B-AS1 was correlated with HCC PD-Ls expression. Finally, PCED1B-AS1 promoted cell proliferation, colony formation and in vivo tumor formation in xenografted nude mice while inhibited apoptosis.

Conclusions PCED1B-AS1 enhances the expression and function of PD-Ls via sponging hsa-miR-194-5p to induce immunosuppression in HCC.

Fei Fan, Keji Chen and Xiaoliang Lu contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12072-020-10101-6) contains supplementary material, which is available to authorized users.

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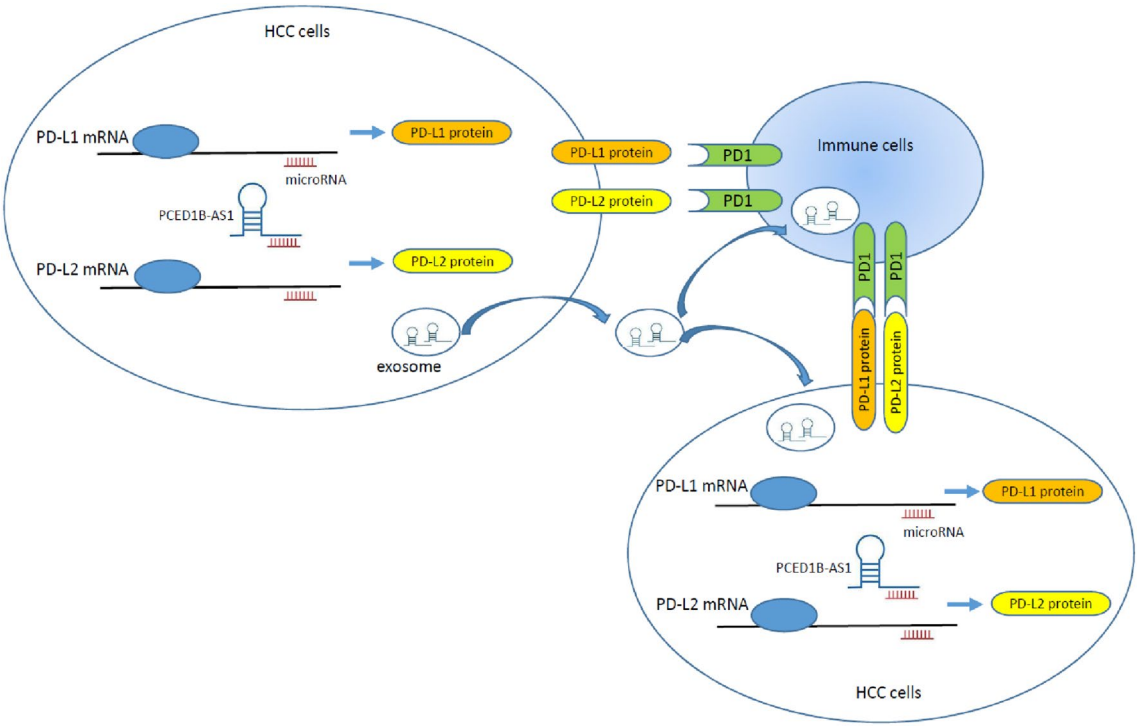
Graphic abstract



Keywords Hepatocellular carcinoma · lncRNA · microRNA · PCED1B-AS1 · miR-194-5p · PD-L1 · PD-L2 · Immune checkpoint · Immunosuppression · Exosome

Background

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults [1]. Although treatments including surgery, radiotherapy, chemotherapy, and targeted therapy like sorafenib are available, the clinical prognosis for patients with advanced HCC remains poor [2]. Fortunately, recent advances in immunotherapy like immune checkpoint (PD-1, PD-L1, or PD-L2) inhibitors bring new hope for cancer treatment [3].

PD-1 is an immune-checkpoint receptor expressed on the surface of immune cells while PD-L1 and PD-L2 are PD-1 ligands (PD-Ls) which are expressed at high levels in many types of tumors. PD-Ls bind to PD-1 to induce apoptosis or inhibition of T cell [4]. In addition to share redundant function, PD-L1 and PD-L2 seem to have similar expression patterns as their expression levels are highly correlated with each other in various tumors [5, 6]. The correlated expression of PD-Ls in tumors suggests that monotherapy targeting PD-L1 or PD-L2 alone may not be sufficient to show significant benefits. It also indicates that PD-L1 and PD-L2 may share upstream regulators. In HCC, PD-L1 and PD-L2 over-expression is significantly associated with poor prognosis [7–9]. However, little is known about how PD-Ls expression is regulated in HCC and its even more intriguing to know whether and how PDL1 and PD-L2 expression is correlated with each other in HCC.

The long non-coding RNAs (lncRNAs) which are non-coding transcripts longer than 200 nt have emerged as important regulators of protein-encoding genes in diverse diseases including cancer [10]. LncRNAs simultaneously regulate multiple target genes via sponging microRNAs [11–13]. In this way, lncRNAs reduce the amount of microRNAs available for the target mRNA and the inhibitory effects of microRNAs on target genes are blocked by lncRNAs. Interestingly, a very recent study demonstrates that lncRNA-microRNA network regulates the expression of immune checkpoint genes in breast cancer [14]. Thus, it gives rise to the possibility that lncRNAs might regulate PD-Ls expression to coordinate immunosuppression in HCC. In this study, we identified lncRNA PCED1B-AS1 as a key regulator for the expression and function of PD-L1 and PD-L2 in HCC. Our results reveal a novel regulatory mechanism of PD-Ls expression by lncRNA-microRNA network in HCC.

Materials and methods

Clinical samples

All cases were confirmed by pathological diagnosis. A total of 45 cases of primary HCC tissues and their adjacent normal tissue were collected freshly during surgery.

Cell cultures

HCC cell lines Huh-7 and HepG2 from ATCC were maintained in Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. CD8 + T cells were isolated from human PBMCs of healthy donors using CD8 + T cell isolation kit (130-096-495, Miltenyi Biotech). Human macrophages were isolated from human PBMCs of healthy donors using MACSprep Chimerism CD14 MicroBeads (130-111-549, Miltenyi Biotech).

PCED1B-AS1 over-expression and knock-down

For over-expression, full sequence of human PCED1B-AS1 (NR_026544.1) was cloned into pLncEXP vector. For PCED1B-AS1 knock-down, two short hairpin RNAs (shRNAs) targeting different sites of human PCED1B-AS1 (NR_026544.1) and scramble control were constructed into the pLentiLox3.7 (pLL3.7) vector. Lenti-virus was packaged and amplified in HEK293T cell. HCC cell lines were infected at an MOI of 5.

Quantitative real-time PCR

Quantitative real time-PCR (qPCR) was performed to measure the relative levels of PD-L1 mRNA, PD-L2 mRNA, lncRNA PCED1B-AS1, and 21-microRNA panel using TaqMan assays. Actin was used as an endogenous control. The relative fold change for each target gene compared to the control group was calculated using the ΔΔCt method.

Western blot

Proteins were extracted from tissues or cell lines using RIPA lysis buffer. Protein samples (50 µg) were resolved by SDS–PAGE and probed with following antibodies: PD-L1 (16-5983-82, eBioscience), PD-L2 (16-5888-82, eBioscience), Akt (4685, CST), p-Akt (4060, CST), P70S6K (2708, CST), p-P70S6K (9205, CST), STAT3 (9139, CST), p-STAT3 (9131, CST), cleaved Notch1 (4147, CST), LRP6 (3395, CST) and p-LRP6 (2568, CST). These antibodies were used at 1:1000 dilution in western blot and used at the final concentration of 10 µg/ml in blocking experiments.

CCK-8 assay

Cell viability of T cells and macrophages was determined by reduction of the tetrazolium salt WST-8 through mitochondrial dehydrogenases using Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) according to the manufacturer's instructions. Cell proliferation of HCC cells was also measured with CCK-8 assay.

Luciferase assay

For 3′UTR luciferase assay of PD-L1 and PD-L2, 1000 bp of human PD-L1 or PD-L2 3′UTR sequence containing binding sites of hsa-mir-194-5p was amplified by PCR and cloned into pMIR-report vector. Renilla luciferase vector was used to normalize for transfection efficiency. HCC cells were transiently transfected with microRNA inhibitor and luciferase vectors using Lipofectamine 2000 for 48 h. Reporter activity was measured by the dual-luciferase assay-system (Promega). The data were presented as fold change relative to the control group.

Bioinformatics prediction

The transcriptome sequencing and microRNA sequencing data of TCGA LIHC dataset were downloaded from UCSC Xena (https://www.xena.ucsc.edu/). Potential microRNAs targeting PD-L1, PD-L2 or PCED1B-AS1 was first predicted by TarPmiR tool (https://www.hulab.ucf.edu/research/projects/miRNA/TarPmiR/) and then validated by RNAhybrid tool (https://www.bibiserv.cebitec.uni-bielefeld.de/rnahybrid/).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. For clinical samples, all data were presented as whiskers-box plots and non-parametric Mann–Whitney *U* test was used for two groups. For cell cultures and mice, all data were presented as mean ± SD and statistical analysis was performed by two-tailed Student's *t* test for two groups and one way ANOVA with Newman–Keuls post hoc test for more than two groups. Spearman's correlation analysis was used to evaluate the correlation between two gene expression levels. Statistically significant difference was defined as $p < 0.05$. For all, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Positive correlations of PCED1B-AS1 with PD-Ls in HCC

We explored RNA sequencing data from TCGA LIHC dataset and found that PD-L1 expression was highly correlated with PD-L2 expression in tumor tissues ($R^2 = 0.58$, $p = 0$, Fig. 1a). This robust correlation from non-biased cohort with large sample size suggests that PD-L1 and PD-L2 have quite similar expression pattern in HCC and it's likely that common regulators simultaneously control the expression of PD-L1 and PD-L2. To search for lncRNAs which regulate PD-L1 and PD-L2 expression at the same time in HCC, we analyzed TCGA LIHC dataset to identify lncRNAs which are correlated with PD-L1 or PD-L2, respectively. The heatmaps show the top 10 lncRNAs which have the highest correlations with PD-L1 (Fig. 1b) or PD-L2 (Fig. 1c), respectively. The exact correlation coefficients and p values were listed in Supplementary Table 1. We found that there was an overlap of four lncRNAs between PD-L1 correlated lncRNAs and PD-L2 correlated lncRNAs: PCED1B-AS1, LOC653653, LOC154761 and LINC00426 (Fig. 1d). It suggests these four lncRNAs might regulate PD-L1 and PD-L2 expression. However, LOC653653, LOC154761, and LINC00426 have pretty low abundance compared to PCED1B-AS1 as their reads were far below 1 TPM in both HCC and normal control tissues (Supplementary Fig. 1A). Interestingly, PCED1B-AS1 was highly correlated with PD-L1 and PD-L2 in HCC tissues (Supplementary Fig. 1B) as well as in normal control tissues (Supplementary Fig. 1C) according to TCGA LIHC data. It suggests that PCED1B-AS1 might be an important regulator of PD-L1 and PD-L2 in HCC. Thus, we focus on PCED1B-AS1 in the current study.

PCED1B-AS1 expression levels were measured by TaqMan assay in HCC tissues ($n = 45$) and matched control tissues ($n = 45$). The results show that PCED1B-AS1 expression levels were higher in HCC tissues compared to matched control tissues (Fig. 1e). This was further supported by PCED1B-AS1 RNA in situ hybridization (RNA-ISH) and the results show that there were intense cytoplasmic signals in HCC sections but much weaker signals in normal control sections (Fig. 1f). Quantification of RNA-ISH shows that HCC tissues had much higher positive rate and quickscore compared to control tissues (Fig. 1g). In addition, the up-regulation of PCED1B-AS1 was independently cross-validated by expression data from TCGA LIHC dataset (Fig. 1h). To demonstrate the correlation of PCED1B-AS1 with PD-L1 or PD-L2, the protein levels of PD-L1 and PD-L2 were measured by western blot in HCC tissues ($n = 45$) and matched control tissues ($n = 45$). The results show that PD-L1 and PD-L2 protein levels were increased in HCC tissues compared to matched control tissues (Fig. 1i) and PD-L1 protein level was positively correlated with PD-L2 protein level (Fig. 1j) in HCC tissues. In addition, PCED1B-AS1 level was also positively correlated with PD-L1 or PD-L2 protein levels, respectively, in HCC tissues (Fig. 1k). Taken together, these results suggest that PCED1B-AS1 is positively correlated with the expression of PD-L1 and PD-L2 in HCC tissues and PCED1B-AS1 up-regulation in HCC might enhance the expression of PD-L1 and PD-L2.

In addition, we analyzed the potential associations of clinico-pathological characteristics with the expression of PD-L1, PD-L2, or PCED1B-AS1 in the same 45 HCC cases in Supplementary Table 3. Further analysis shows that high PD-L1 expression in HCC tissue was associated with large tumor size and TNM stage III–IV, high PD-L2 expression in HCC tissue was associated with large tumor size and TNM stage III–IV, and high PCED1B-AS1 expression in HCC tissue was associated with multiple tumor numbers, large tumor size, and TNM stage III–IV. More importantly, high PD-L1 expression and high PCED1B-AS1 expression were associated with poor survival, respectively (Supplementary Table 4). These results further support that PCED1B-AS1 plays an important role in the pathogenesis of HCC.

Negative correlation of PCED1B-AS1 with PD-Ls targeting microRNA in HCC

Next, we tried to understand whether and how could PCED1B-AS1 regulate PD-L1 and PD-L2 expression. Nuclear lncRNAs can directly interact with chromatin to regulate the transcription of target genes while cytoplasmic lncRNAs can act as a sponge of microRNAs to modulate gene expression. Thus, lncRNAs regulate gene expression via distinct mechanisms, depending on their subcellular localization. RNA ISH in tissue sections shows that PCED1B-AS1 was localized in the cytosol (Fig. 1f). To further confirm this, we performed RNA ISH in 2 HCC cell lines (Huh-7 and HepG2). The results show that PCED1B-AS1 was dominantly localized in cytosol in HCC cell lines (Fig. 2a). In addition, we performed subcellular fractionation in HCC cell lines and measured PCED1B-AS1 levels in nuclear and cytoplasmic fractions with TaqMan assay. The results show that most PCED1B-AS1 had a cytoplasmic distribution (Fig. 2b). It suggests that PCED1B-AS1 may act as a sponge of microRNAs in the cytosol to regulate PD-Ls expression.

To identify the microRNAs mediating the interaction between PCED1B-AS1 and PD-Ls, we used TarPmiR [15] and RNAhybrid [16] to predict microRNAs targeting PCED1B-AS1, PD-L1 or PD-L2, respectively

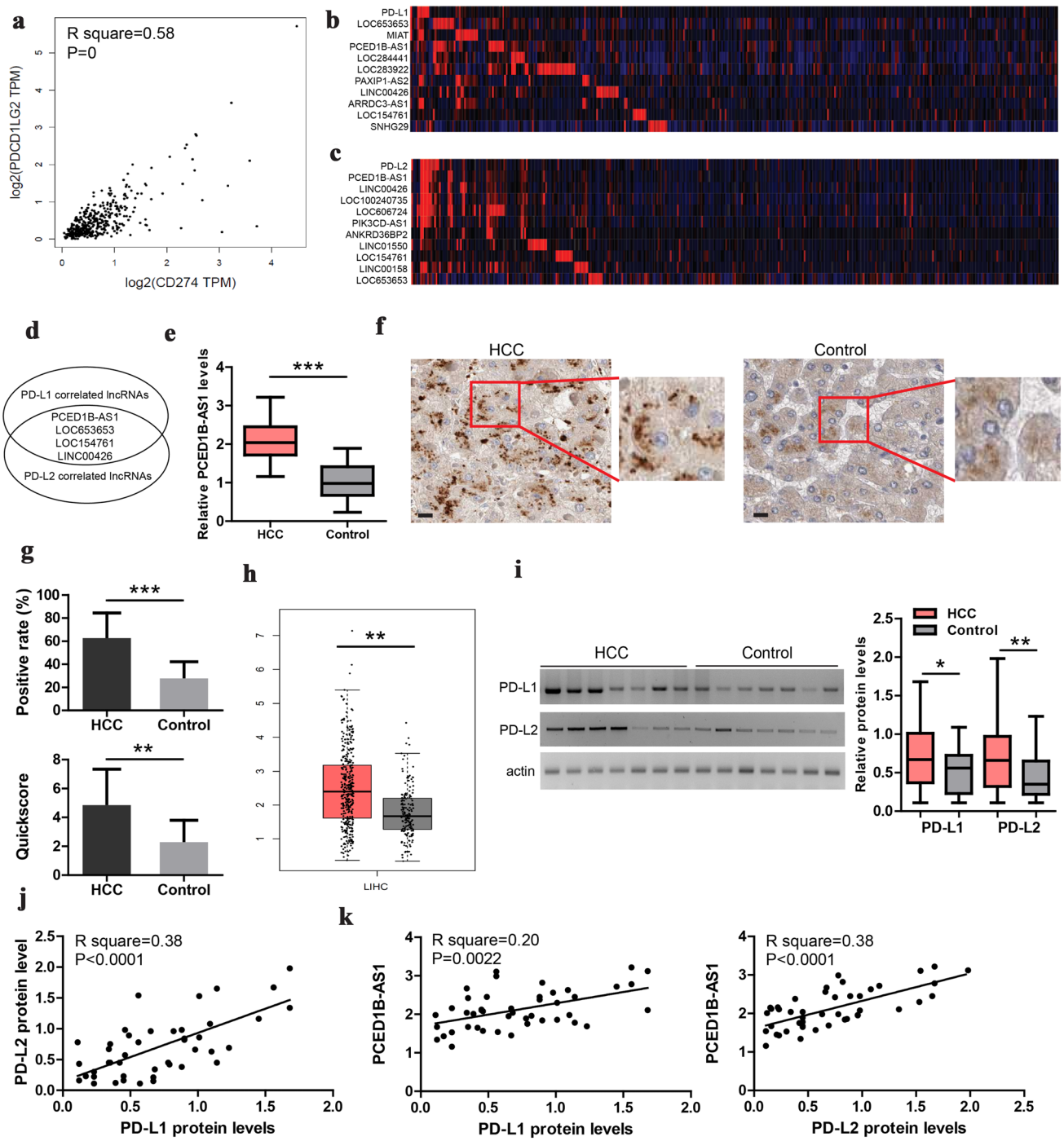
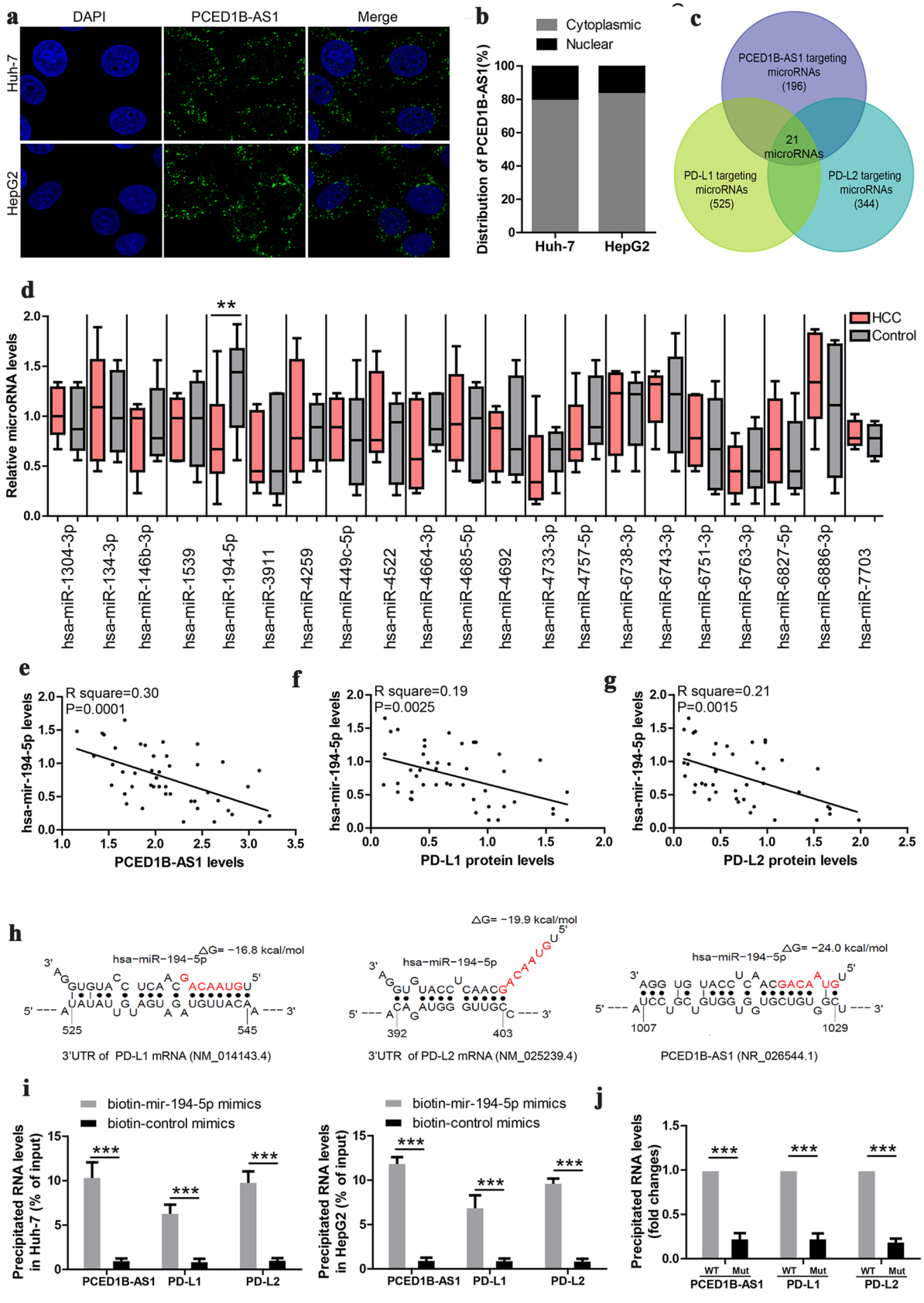


Fig. 1 Positive correlations of PCED1B-AS1 with PD-Ls in HCC. **a** Scatter plot showing the positive correlation between PD-L1 (CD274) and PD-L2 (PDCD1LG2) in tumor tissues from TCGA LIHC dataset ($R^2=0.58$, $p=0$). **b** Heatmap showing the expression levels of PD-L1 and its correlated lncRNAs in tumor tissues from TCGA LIHC dataset. **c** Heatmap showing the expression levels of PD-L2 and its correlated lncRNAs in tumor tissues from TCGA LIHC dataset. **d** Overlap of PD-L1 correlated lncRNAs and PD-L2 correlated lncRNAs in tumor tissues from TCGA LIHC dataset. **e** TaqMan assay showing expression level of lncRNA PCED1B-AS1 in HCC tissues ($n=45$) and matched control tissues ($n=45$). Data were presented as whiskers-box plots. **f** Representative images of RNA ISH showing PCED1B-AS1 expression in sections from HCC tissues ($n=45$) and matched control tissues ($n=45$). Scale bar = 30um. **g** Positive rate and Quickscores of PCED1B-AS1 RNA ISH in HCC tissues ($n=45$) and matched control tissues ($n=45$). **h** Expression levels of PCED1B-AS1 in TCGA LIHC dataset. **i** Western blots of PD-L1 and PD-L2 in HCC tissues ($n=45$) and matched control tissues ($n=45$). **j** Scatter plot showing the positive correlation between PD-L1 protein level and PD-L2 protein level in HCC tissues ($R^2=0.38$, $p<0.0001$). **k** Scatter plots showing the positive correlations of PCED1B-AS1 expression level with PD-L1 protein level ($R^2=0.20$, $p=0.0022$) or PD-L2 protein level ($R^2=0.38$, $p<0.0001$) in HCC tissues. For all, $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$


a
DAPI
PCED1B-AS1
Merge
Huh-7
HepG2
b
Cytoplasmic
Nuclear
Distribution of PCED1B-AS1(%)
Huh-7
HepG2
c
PCED1B-AS1 targeting microRNAs (196)
21 microRNAs
PD-L1 targeting microRNAs (525)
PD-L2 targeting microRNAs (344)
d
Relative microRNA levels
HCC
Control
**
hsa-miR-1304-3p
hsa-miR-134-3p
hsa-miR-146b-3p
hsa-miR-1539
hsa-miR-194-5p
hsa-miR-3911
hsa-miR-4259
hsa-miR-449c-5p
hsa-miR-4522
hsa-miR-4664-3p
hsa-miR-4685-5p
hsa-miR-4692
hsa-miR-4733-3p
hsa-miR-4757-5p
hsa-miR-6738-3p
hsa-miR-6743-3p
hsa-miR-6751-3p
hsa-miR-6763-3p
hsa-miR-6827-5p
hsa-miR-6886-3p
hsa-miR-7703
e
R square=0.30
P=0.0001
hsa-mir-194-5p levels
PCED1B-AS1 levels
f
R square=0.19
P=0.0025
hsa-mir-194-5p levels
PD-L1 protein levels
g
R square=0.21
P=0.0015
hsa-mir-194-5p levels
PD-L2 protein levels
h
hsa-miR-194-5p
ΔG= −16.8 kcal/mol
525
545
3'UTR of PD-L1 mRNA (NM_014143.4)
ΔG= −19.9 kcal/mol
392
403
3'UTR of PD-L2 mRNA (NM_025239.4)
ΔG= −24.0 kcal/mol
1007
1029
PCED1B-AS1 (NR_026544.1)
i
biotin-mir-194-5p mimics
biotin-control mimics
Precipitated RNA levels in Huh-7 (% of input)
Precipitated RNA levels in HepG2 (% of input)
PCED1B-AS1
PD-L1
PD-L2
j
Precipitated RNA levels (fold changes)
WT
Mut
PCED1B-AS1
PD-L1
PD-L2

◂**Fig. 2** Negative correlation of PCED1B-AS1 with PD-Ls targeting microRNA in HCC. **a** RNA ISH showing PCED1B-AS1 expression in Huh-7 and HepG2 cells. **b** TaqMan assay showing subcellular distribution of PCED1B-AS1 in Huh-7 and HepG2 cells. **c** Venn diagram showing the overlap of PD-L1 targeting microRNAs, PD-L2 targeting microRNAs and PCED1B-AS1 targeting microRNAs. **d** TaqMan assay showing the expression levels of 21-microRNA panel in HCC tissues ($n=45$) and matched control tissues ($n=45$). Data were presented as whiskers-box plots. Scatter plots showing the negative correlation of hsa-mir-194-5p with PCED1B-AS1 (**e** $R^2=0.30$, $p=0.0001$), PD-L1 protein level (**f** $R^2=0.19$, $p=0.0025$) or PD-L2 protein level (**g** $R^2=0.21$, $p=0.0015$) in HCC tissues. **h** Predicted binding sites of hsa-mir-194-5p on PD-L1 3′UTR, PD-L2 3′UTR and PCED1B-AS1. **i** Biotinylated microRNA pulldown assay showing the precipitated PD-L1, PD-L2 and PCED1B-AS1 RNA transcripts by indicated biotinylated mimics in Huh-7 and HepG2 cells. **j** In vitro RNA binding assay showing the interaction of hsa-mir-194-5p with wild-type or mutant RNA transcripts of PD-L1, PD-L2 or PCED1B-AS1. For all, $^{**}p<0.01$; $^{***}p<0.001$

(Supplementary Fig. 2A). The prediction shows that 196 microRNAs target PCED1B-AS1, 525 microRNAs target PD-L1 and 344 microRNAs target PD-L2 (Supplementary Table 2). There was an overlap of 21 microRNAs among the above three lists (Fig. 2c). We measured the expression of the 21-microRNA panel by TaqMan assay in HCC tissues ($n=45$) and matched control tissues ($n=45$). The results show that hsa-mir-194-5p level was significantly reduced in HCC tissues compared to matched control tissues (Fig. 2d). In addition, hsa-mir-194-5p level was negatively correlated with PCED1B-AS1 level (Fig. 2e), PD-L1 protein level (Fig. 2f) or PD-L2 protein level (Fig. 2g), respectively, in HCC tissues. Consistently, microRNA sequencing data from TCGA LIHC dataset show that hsa-mir-194-5p level was negatively correlated with PD-L1 mRNA level (Supplementary Fig. 2B), PD-L2 mRNA level (Supplementary Fig. 2C) or PCED1B-AS1 level (Supplementary Fig. 2D). These results suggest that hsa-mir-194-5p might interact with RNA transcript of PCED1B-AS1, PD-L1, or PD-L2, respectively, and the predicted binding sites were shown in Fig. 2h.

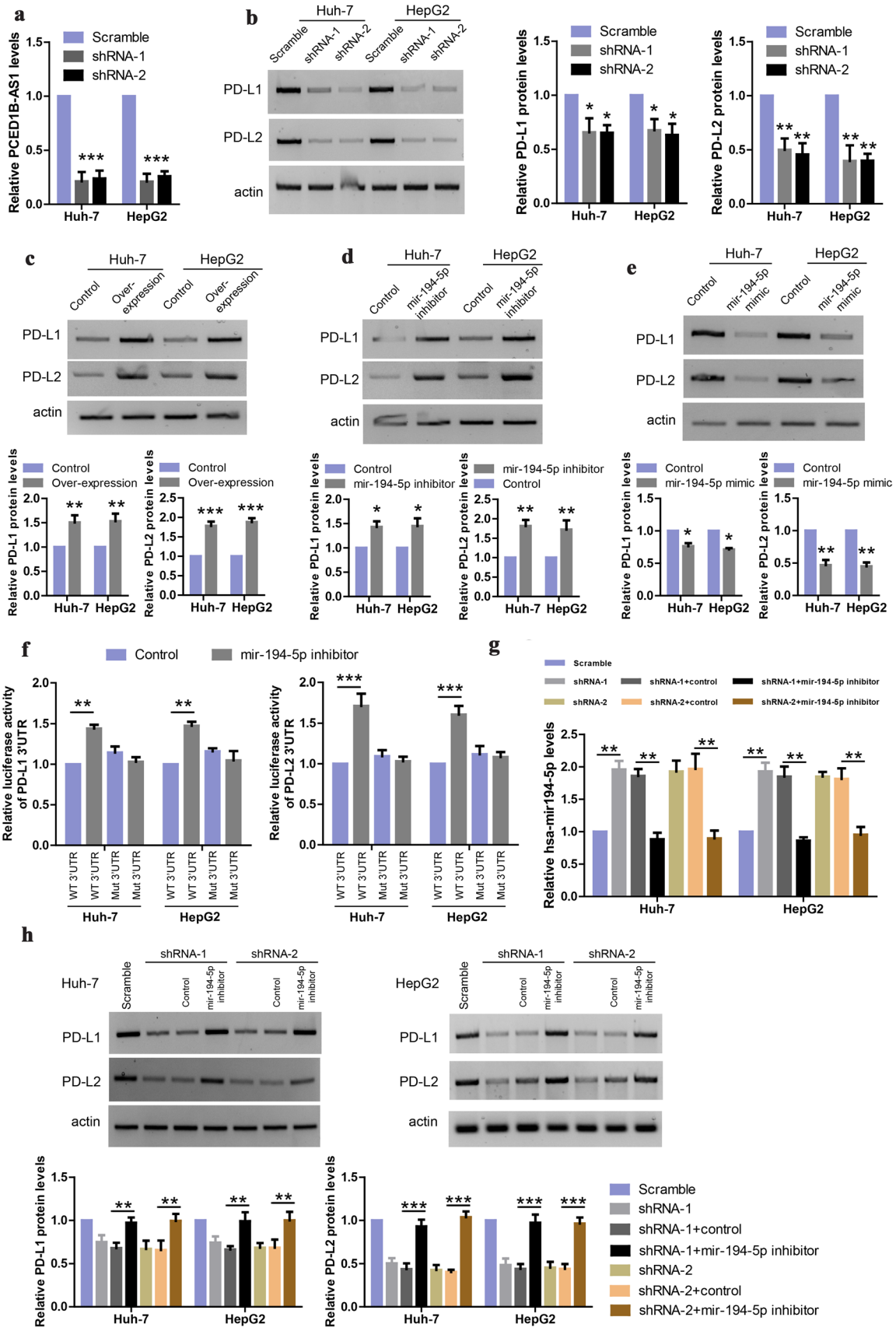
To provide experimental evidence that hsa-mir-194-5p interacts with RNA transcript of PCED1B-AS1, PD-L1 or PD-L2, we performed RNA pulldown with biotin labeled microRNA mimics in HCC cell lines. Cells were transfected with biotinylated hsa-mir-194-5p mimics or biotinylated control mimics for 48 h. Then, cells were lyzed and the biotinylated mimics were isolated by streptavidin magnetic beads. The RNA transcripts of PCED1B-AS1, PD-L1, or PD-L2 co-precipitated with biotinylated mimics were detected by TaqMan assay. The results show that RNA transcripts of PCED1B-AS1, PD-L1, and PD-L2 were co-precipitated with biotinylated hsa-mir-194-5p mimics but not with biotinylated control mimics in both HCC cell lines (Fig. 2i). To further confirm the interaction of hsa-mir-194-5p with PCED1B-AS1, PD-L1, or PD-L2 RNA transcript, we produced 500 nt fragment of PCED1B-AS1, PD-L1, or PD-L2 RNA transcripts covering the predicted binding sites by in vitro transcription. After incubating RNA fragments with biotinylated hsa-mir-194-5p mimics, RNA pulldown was performed with streptavidin magnetic beads and co-precipitated RNA fragments were detected by TaqMan assay. The results show that wild-type RNA fragments of PCED1B-AS1, PD-L1 and PD-L2 were co-precipitated with biotinylated hsa-mir-194-5p mimics while mutations of predicted binding sites in the RNA fragments abolished the binding of biotinylated hsa-mir-194-5p mimics to these RNA fragments (Fig. 2j). Based on the correlations and interactions of hsa-mir-194-5p with PCED1B-AS1 and PD-Ls, we hypothesized that PCED1B-AS1 could enhance the expression of PD-L1 and PD-L2 via sponging hsa-mir-194-5p in HCC.

PCED1B-AS1 sponges hsa-mir-194-5p to enhance the expression of PD-L1 and PD-L2

To directly demonstrate that PCED1B-AS1 regulates the expression of PD-L1 and PD-L2, PCED1B-AS1 knockdown was performed using lenti-viral shRNAs in 2 HCC cell lines (Fig. 3a) and the protein levels of PD-L1 and PD-L2 were measured by western blot (Fig. 3b). The results show that PCED1B-AS1 knockdown reduced the protein levels of PD-L1 and PD-L2 at the same time. In addition, lenti-viral over-expression of PCED1B-AS1 increased the protein levels of PD-L1 and PD-L2 at the same time (Fig. 3c). These results suggest that PCED1B-AS1 controls the expression of PD-L1 and PD-L2 in HCC.

To directly demonstrate that hsa-mir-194-5p regulates the expression of PD-L1 and PD-L2, mimic or inhibitor of hsa-mir-194-5p was transfected in HCC cell lines for 48 h and the protein levels of PD-L1 and PD-L2 were measured by western blot. The results show that hsa-mir-194-5p inhibitor increased the protein levels of PD-L1 and PD-L2 (Fig. 3d) while hsa-mir-194-5p mimic reduced the protein levels of PD-L1 and PD-L2 (Fig. 3e). To further support that PD-L1 and PD-L2 are direct targets of hsa-mir-194-5p, luciferase assays were performed in HCC cell lines. In cells transfected with luciferase reporter vector containing PD-L1 3′UTR, co-transfection with hsa-mir-194-5p inhibitor enhanced the luciferase activity while mutation of predicted binding site abolished the effects of hsa-mir-194-5p inhibitor (Fig. 3f). Similar results were obtained form cells transfected with luciferase reporter vector containing PD-L2 3′UTR. Taken together, these results suggest that hsa-mir-194-5p targets PD-L1 and PD-L2 to inhibit their expression in HCC.

To directly demonstrate that the effects of PCED1B-AS1 on PD-Ls expression are mediated by hsa-mir-194-5p, hsa-mir-194-5p inhibitor was transfected in HCC cell lines after PCED1B-AS1 knockdown. The results show that PCED1B-AS1 knockdown increased hsa-mir-194-5p level (Fig. 3g)


a
Relative PCED1B-AS1 levels
Scramble
shRNA-1
shRNA-2
Huh-7
HepG2
b
Huh-7
HepG2
Scramble
shRNA-1
shRNA-2
PD-L1
PD-L2
actin
Relative PD-L1 protein levels
Relative PD-L2 protein levels
c
Control
Over-expression
d
Control
mir-194-5p inhibitor
e
Control
mir-194-5p mimic
f
Relative luciferase activity of PD-L1 3'UTR
Relative luciferase activity of PD-L2 3'UTR
WT 3'UTR
Mut 3'UTR
g
Scramble
shRNA-1
shRNA-2
shRNA-1+control
shRNA-2+control
shRNA-1+mir-194-5p inhibitor
shRNA-2+mir-194-5p inhibitor
Relative hsa-mir194-5p levels
h

◂**Fig. 3** PCED1B-AS1 sponges hsa-mir-194-5p to enhance the expression of PD-L1 and PD-L2. **a** TaqMan assay showing endogenous PCED1B-AS1 levels after lentiviral knockdown of PCED1B-AS1 in Huh-7 and HepG2 cells. **b** Western blots showing the protein levels of PD-L1 and PD-L2 after PCED1B-AS1 knockdown in Huh-7 and HepG2 cells. **c** Western blots showing the protein levels of PD-L1 and PD-L2 after PCED1B-AS1 over-expression in Huh-7 and HepG2 cells. **d** Western blots showing the protein levels of PD-L1 and PD-L2 after hsa-mir-194-5p inhibitor transfection in Huh-7 and HepG2 cells. **e** Western blots showing the protein levels of PD-L1 and PD-L2 after hsa-mir-194-5p mimic transfection in Huh-7 and HepG2 cells. **f** Luciferase assays showing the effects of hsa-mir-194-5p inhibitor on wild-type or mutant PD-L1 3-UTR and PD-L2 3′UTR, respectively, in Huh-7 and HepG2 cells. **g** TaqMan assay showing endogenous hsa-mir-194-5p levels after PCED1B-AS1 knockdown and co-transfection of hsa-mir-194-5p inhibitor in Huh-7 and HepG2 cells. **h** Western blots showing the protein levels of PD-L1 and PD-L2 after PCED1B-AS1 knockdown and co-transfection of hsa-mir-194-5p inhibitor in Huh-7 and HepG2 cells. For all, $*p<0.05$; $**p<0.01$; $***p<0.001$

but reduced PD-L1 and PD-L2 protein levels (Fig. 3h). Meanwhile, co-transfection of hsa-mir-194-5p inhibitor restored PD-L1 and PD-L2 protein levels. Taken together, these results suggest that PCED1B-AS1 sponges hsa-mir-194-5p to enhance the expression of PD-L1 and PD-L2 in HCC.

HCC cells release and uptake PCED1B-AS1 containing exosomes

As tumor cells release exosomes which are rich in various contents including lncRNAs to modulate the behaviors of receipt cells, we tried to determine whether HCC cells could release and uptake PCED1B-AS1 via exosomes. Exosomes were purified from the supernatant of HepG2 cell culture and the size distribution of purified exosomes was between 100 and 200 nm (Fig. 4a). Western blots show that exosomes markers like CD9, CD63, and CD81 were enriched in purified exosomes (Fig. 4b). Then, we explored whether HCC cells could uptake the purified exosomes. Huh-7 and HepG2 cells were incubated with purified exosomes which were labeled by green fluorescent dye PKH67. Then, HCC cells were imaged and the results show that labeled exosomes were located in the cytosol of Huh-7 and HepG2 cells (Fig. 4c). In addition, PCED1B-AS1 level within HepG2-derived exosomes was measured by TaqMan assay. The results show that PCED1B-AS1 was detectable in exosomes derived from HepG2 cells infected with scramble control while PCED1B-AS1 level was greatly reduced in exosomes derived from HepG2 cells infected with PCED1B-AS1 shRNAs (Fig. 4d). Taken together, these results suggest HCC cells release and uptake PCED1B-AS1 containing exosomes.

To investigate whether PCED1B-AS1 containing exosomes could modulate gene expression in receipt HCC cells, Huh-7 and HepG2 cells were incubated exosomes at indicated dose and the expression of hsa-mir-194-5p was measured by TaqMan assay. The results show that PCED1B-AS1 containing exosomes reduced hsa-mir-194-5p level in a dose-dependent manner in HCC cell lines (Fig. 4e). In addition, PD-L1 and PD-L2 expression was measured by western blot and the results show that PCED1B-AS1 containing exosomes increased PD-L1 and PD-L2 protein levels in a dose-dependent manner in receipt HCC cells (Fig. 4f). To confirm that PCED1B-AS1 is responsible for the effects of purified exosomes, exosomes derived from HepG2 cells infected with scramble, PCED1B-AS1 shRNA1, or shRNA2 were used to incubate HCC cells. The results show that exosomes from scramble infected cells reduced hsa-mir-194-5p level (Fig. 4g) and increased the protein levels of PD-L1 and PD-L2 (Fig. 4h). In contrast, exosomes from PCED1B-AS1 shRNA infected cells had no such effect. Taken together, these results suggest HCC cells release exosomal PCED1B-AS1 to regulate gene expression of receipt HCC cells.

We also investigated whether exosomal PCED1B-AS1 is detectable in the blood samples from the same 45 HCC patients. Plasma exosomes were extracted and exosomal PCED1B-AS1 was measured by TaqMan assay. The results show that blood PCED1B-AS1 level had a high correlation with PCED1B-AS1 in HCC tissues ($R^2=0.53$, $p<0.0001$, Fig. 4i), suggesting that HCC tissues are the major source of blood exosomal PCED1B-AS1 in these patients. In addition, blood exosomal PCED1B-AS1 level had a significant correlation with PD-L1 protein level ($R^2=0.16$, $p=0.006$) and PD-L2 ($R^2=0.33$, $p<0.0001$) in HCC tissues. Further analysis shows that high plasma exosomal PCED1B-AS1 level was associated with TNM stage III–IV (Supplementary Table 3). In addition, high plasma exosomal PCED1B-AS1 level was associated with poor survival (Supplementary Table 4). Taken together, it suggests that blood exosomal PCED1B-AS1 might be a non-invasive biomarker to monitor PD-Ls expression in HCC patients.

PCED1B-AS1 induces immunosuppression of immune cells

As PCED1B-AS1 enhanced PD-Ls expression, it is likely that PCED1B-AS1 could induce immunosuppression in HCC. Thus, we used an in vitro co-culture system in which human primary T cells were co-cultured with HCC cell lines over-expressing PCED1B-AS1. The apoptosis of co-cultured T cells was measured by Annexin V-PE FACS assay and Caspase-Glo 3/7 assay, respectively. Annexin V-PE assay shows that co-culture with HCC cells over-expressing PCED1B-AS1 increased early apoptotic T cells while co-incubation with PD-L1 and PD-L2 blocking antibodies eliminated this effect (Fig. 5a). Caspase-Glo 3/7 assay shows that co-culture with HCC cells over-expressing PCED1B-AS1

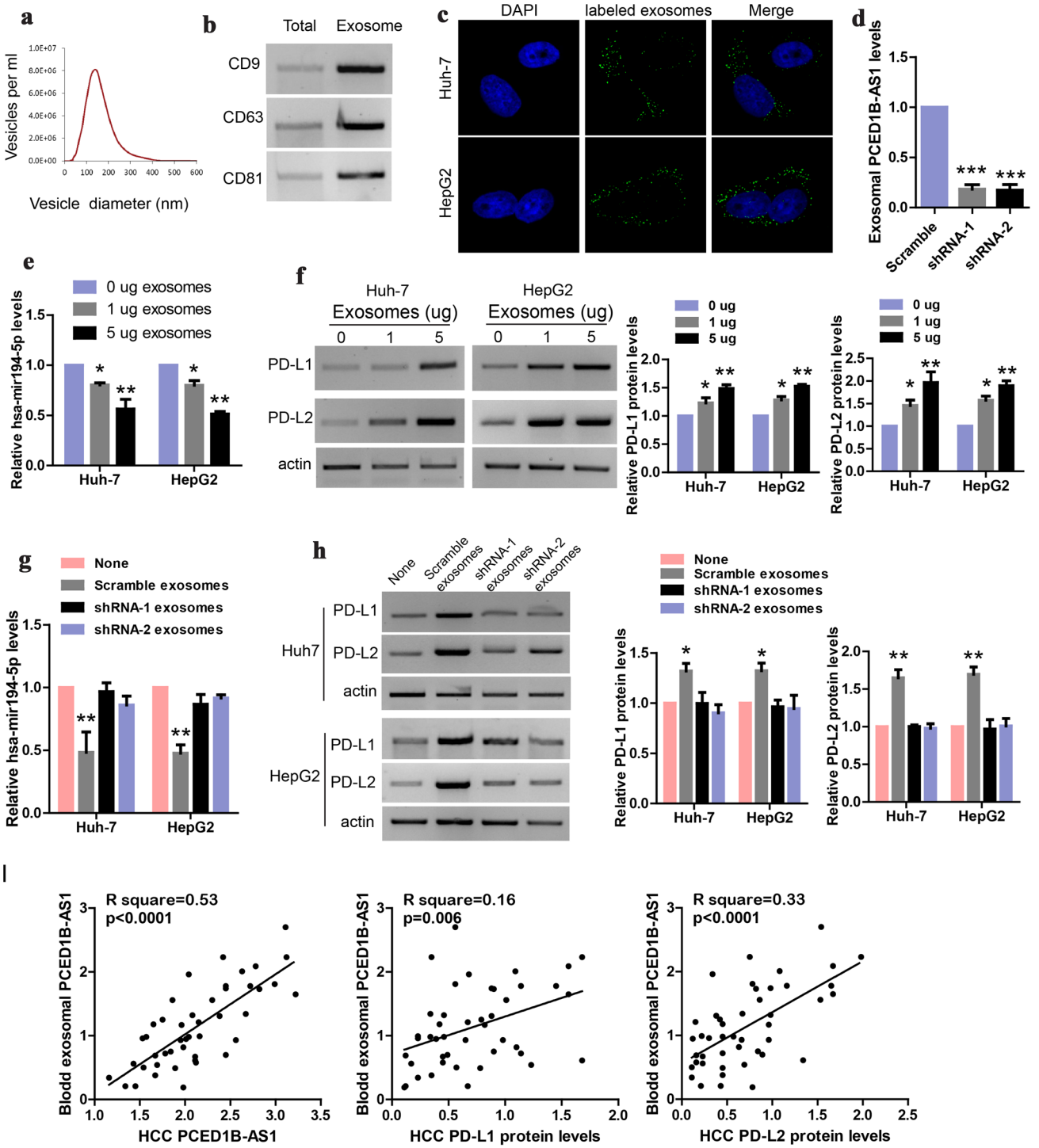


Fig. 4 HCC cells release and uptake PCED1B-AS1 containing exosomes. **a** Size distribution of isolated exosomes from HepG2 cells. **b** Western blots showing the enrichment of exosome markers (CD9, CD63, and CD81) in isolated exosomes. **c** Uptake of PKH67 labeled exosomes by Huh-7 or HepG2 cells. **d** TaqMan assay showing PCED1B-AS1 levels in exosomes derived from HepG2 cells after PCED1B-AS1 knockdown. **e** TaqMan assays showing dose-dependent effects of PCED1B-AS1 containing exosomes on endogenous hsa-mir-194-5p levels in receipt Huh-7 and HepG2 cells. **f** Western blots showing dose-dependent effects of PCED1B-AS1 containing exosomes on endogenous PD-L1 and PD-L2 protein levels in receipt Huh-7 and HepG2 cells. **g** TaqMan assays showing the endogenous hsa-mir-194-5p levels in receipt Huh-7 and HepG2 cells treated with exosomes isolated from HepG2 cells after PCED1B-AS1 knockdown. **h** Western blots showing endogenous PD-L1 and PD-L2 protein levels in receipt Huh-7 and HepG2 cells treated with exosomes isolated from HepG2 cells after PCED1B-AS1 knockdown. **i** Scatter plots showing the positive correlations of blood exosomal PCED1B-AS1 level with HCC PCED1B-AS1 level ($R^2=0.53$, $p<0.0001$), HCC PD-L1 protein level ($R^2=0.16$, $p=0.006$) or HCC PD-L2 protein level ($R^2=0.33$, $p<0.0001$). For all, $*p<0.05$; $**p<0.01$; $***p<0.001$

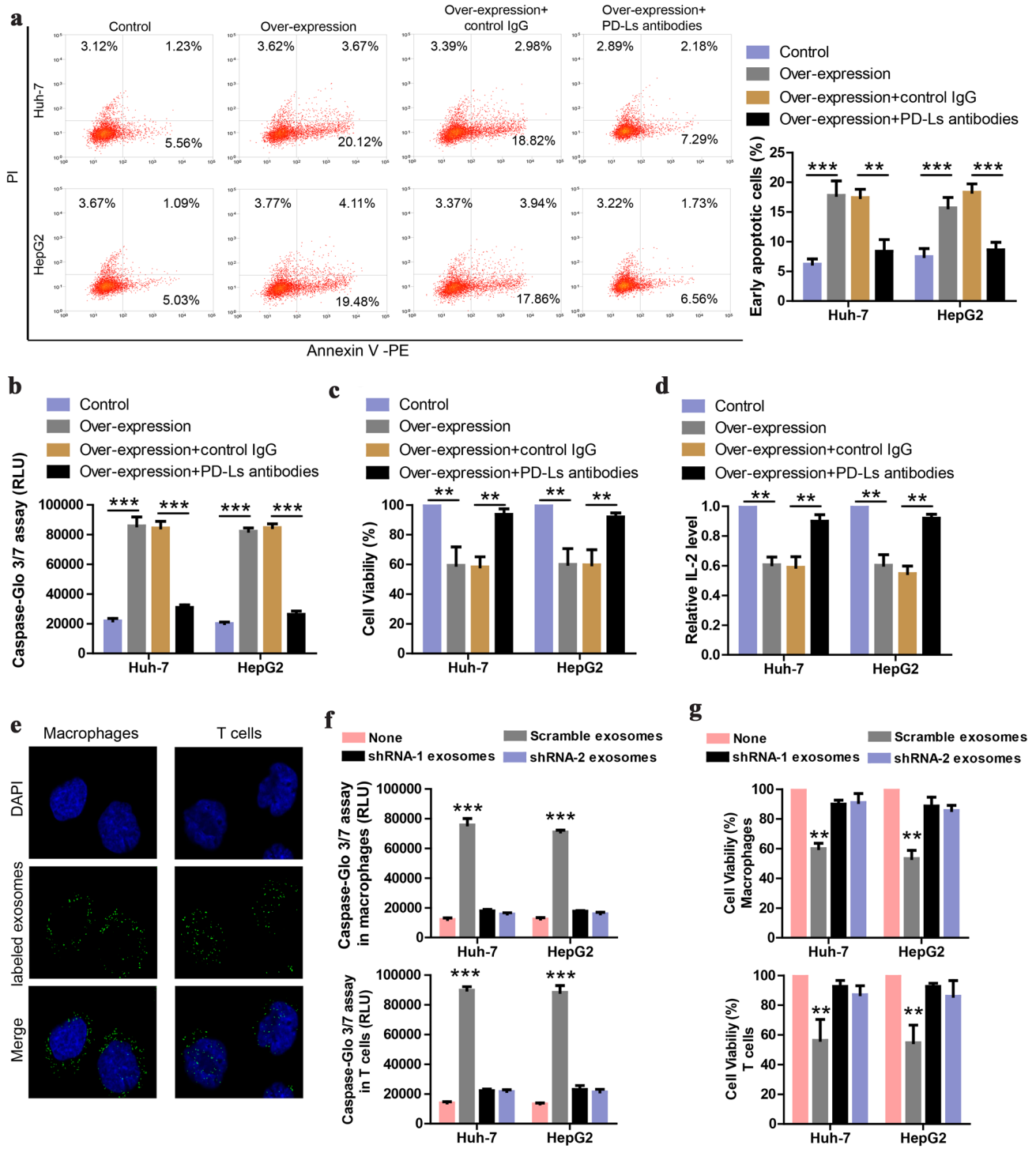


Fig. 5 PCED1B-AS1 induces immunosuppression of immune cells. **a** Annexin V-PE apoptosis assay showing apoptosis of human T cells after co-cultured with Huh-7 or HepG2 cells over-expressing PCED1B-AS1 and treated with the combination of PD-L1 antibody and PD-L2 antibody (PD-Ls antibodies). **b** Caspase-Glo 3/7 assay showing caspase activity of human T cells after co-cultured with Huh-7 or HepG2 cells over-expressing PCED1B-AS1 and treated with PD-Ls antibodies. **c** CCK-8 assay showing cell viability of human T cells after co-cultured with Huh-7 or HepG2 cells over-expressing PCED1B-AS1 and treated with PD-Ls antibodies. **d** ELISA results showing IL2 levels in the culture medium of human T cells after co-cultured with Huh-7 or HepG2 cells over-expressing PCED1B-AS1 and treated with PD-Ls antibodies. **e** Uptake of PKH67 labeled exosomes by human macrophages and T cells. **f** Caspase-Glo 3/7 assay showing caspase activity of human macrophages and T cells treated with exosomes isolated from HepG2 cells after PCED1B-AS1 knockdown. **g** CCK-8 assay showing cell viability of human macrophages and T cells treated with exosomes isolated from HepG2 cells after PCED1B-AS1 knockdown. For all, $^{**}p<0.01$; $^{***}p<0.001$

enhanced caspase activity in T cells while co-incubation with PD-L1 and PD-L2 blocking antibodies eliminated this effect (Fig. 5b). T-cell viability after co-culture was measured by CCK-8 assay and the results show that co-culture with HCC cells over-expressing PCED1B-AS1 impaired T-cell viability while co-incubation with PD-L1 and PD-L2 blocking antibodies eliminated this effect (Fig. 5c). In addition, T cells secrete Interleukin 2 (IL2) which is essential for T-cell proliferation and activation and IL2 secretion is therefore a marker of T-cell activity [17, 18]. Thus, IL2 secretion by T cells after co-culture was measured by ELISA and the results show that co-culture with HCC cells over-expressing PCED1B-AS1 impaired IL2 secretion of T cells while co-incubation with PD-L1 and PD-L2 blocking antibodies eliminated this effect (Fig. 5d). Taken together, these results suggest that PCED1B-AS1 expression in HCC induces PD-Ls-mediated immunosuppression in co-cultured T cells.

Next, we asked whether immune cells like T cells and macrophages are able to uptake PCED1B-AS1 containing exosomes from HCC cells. Human primary T cells and macrophages were isolated, incubated with labeled exosomes before imaging. The results show that labeled exosomes were mainly located in the cytosol of macrophages and T cells (Fig. 5e). Then, we measured the functional effects of PCED1B-AS1 containing exosomes on macrophages and T cells. Caspase-Glo 3/7 assay shows that exosomes from scramble infected HCC cells activated caspase activity in macrophages and T cells while exosomes from PCED1B-AS1 knockdown HCC cells had no such effect (Fig. 5f). CCK-8 assay shows that exosomes from scramble infected HCC cells impaired cell viability of macrophages and T cells while exosomes from PCED1B-AS1 knockdown HCC cells had no such effect (Fig. 5g).

PCED1B-AS1 promotes aggressive tumor behaviors in in vitro and in vivo assays

Recent study shows that PCED1B-AS1 also activates proliferation and inhibits apoptosis in gliomas [19] and it suggests that PCED1B-AS1 may have a broad influence on HCC tumor behaviors beyond immunosuppresion. Thus, we investigated the effects of PCED1B-AS1 knock-down in Huh-7 cell line in following in vitro and in vivo assays: cell proliferation, colony formation, Caspase-Glo 3/7 assay and in vivo tumor formation. The results show that PCED1B-AS1 knock-down inhibited cell proliferation in CCK-8 assay (Fig. 6a), reduced the number of colony (Fig. 6b) and enhanced caspase activity (Fig. 6c). In subcutaneously xenografted model, five nude mice were randomly assigned to each group: Scramble ($n=5$), PCED1B-AS1 shRNA1 ($n=5$) and PCED1B-AS1 shRNA2 ($n=5$). Four weeks after implantation, mice were sacrificed and the tumor tissues were collected. The results show that tumors from PCED1B-AS1 shRNA1 or shRNA2 group had much smaller size compared to Scramble group (Fig. 6d). Taken together, these results suggest that PCED1B-AS1 promotes aggressive tumor phenotypes in HCC.

Next, we explored the possible signaling pathway underlying the oncogenic effects of PCED1B-AS1 in HCC. We notice that PD-L1 has diverse immune-independent functions in tumors. For example, PD-L1 could translocate into nucleus to regulate gene expression and facilitate tumor necrosis [20]. PD-L1 has antiapoptotic effects in cancer cell death induced by FAS pathway or Staurosporine [21]. In addition, PD-L1 regulates proliferation and autophagy via mTOR signaling [22]. Taken together with our finding that PCED1B-AS1 regulates PD-Ls expression in HCC, it's likely that PD-L1 may mediate the oncogenic effects of PCED1B-AS1 and we further tested several potential downstream signaling pathways by western blots in Huh-7 cell line after PCED1B-AS1 knockdown. The results show that mTOR signaling proteins like p-P70S6K^{T389} and p-Akt^{S473} were reduced by PCED1B-AS1 knockdown (Fig. 6e). In contrast, pathways like Wnt (total LRP6 and p-LRP6), STAT (total STAT3 and p-STAT3), Notch (cleaved Notch1) were not affected by PCED1B-AS1 knockdown. These results were further confirmed in tumor tissues from xenograft nude mice (Fig. 6f). Taken together, these new results suggest that mTOR signaling may mediate the oncogenic effects of PCED1B-AS1 in HCC.

Discussion

Here, we report that PD-L1 expression was highly correlated with PD-L2 expression in HCC, suggesting that their expression may be controlled by the same regulators. Using bioinformatics analysis of TCGA LIHC dataset, we find that lncRNA PCED1B-AS1 was correlated with PD-Ls and PD-Ls targeting hsa-mir-194-5p. These correlations were confirmed experimentally in clinical samples. Then, we further elucidated the roles of PCED1B-AS1 in HCC (Fig. 6g). Our results support that PCED1B-AS1 enhanced the expression of PD-L1 and PD-L2 in HCC via sponging hsa-mir-194-5p, and PCED1B-AS1-induced PD-Ls-mediated immunosuppression in co-cultured T cells. In addition, HCC cells released PCED1B-AS1 containing exosomes and the exosomal PCED1B-AS1 could regulate PD-Ls expression in receipt HCC cells while inhibit receipt T cells and macrophages. Finally, we show that PCED1B-AS1 promoted aggressive tumor behaviors in in vitro and in vivo assays. The oncogenic effects of PCED1B-AS1 in our study are supported by two recent studies in gliomas [19, 23]. Taken together, our study reveals a novel regulatory network of

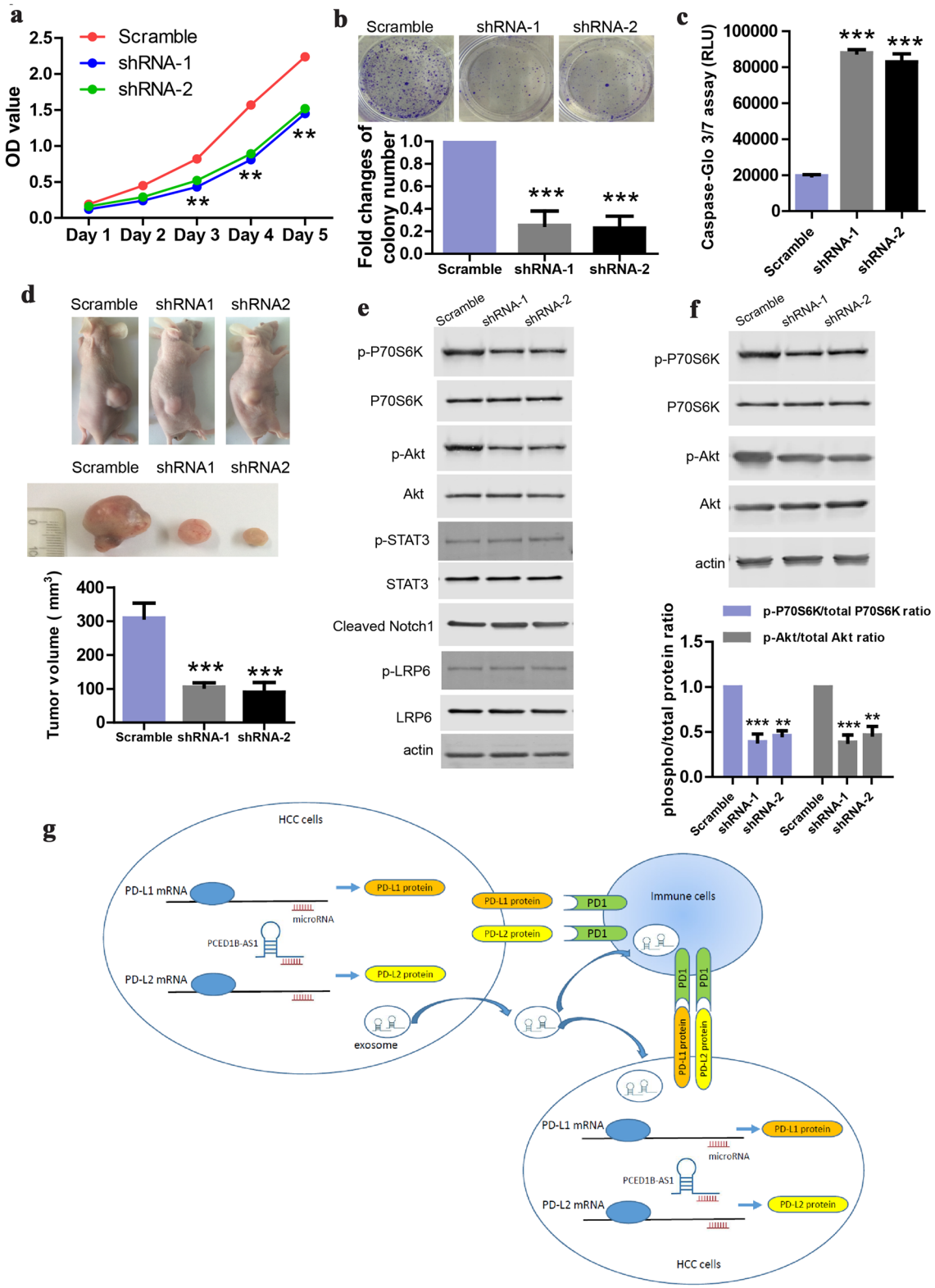


Fig. 6 PCED1B-AS1 promotes aggressive tumor behaviors in in vitro and in vivo assays. **a** CCK-8 assay showing cell proliferation of Huh-7 cells after PCED1B-AS1 knockdown. **b** Colony formation assay in Huh-7 cells after PCED1B-AS1 knockdown. **c** Caspase-Glo 3/7 assay showing caspase activity in Huh-7 cells after PCED1B-AS1 knockdown. **d** Representative images and quantification of tumor size in nude mice xenografted with Huh-7 cells after PCED1B-AS1 knockdown. **e** Western blots showing indicated protein levels in Huh-7 cells after PCED1B-AS1 knockdown. **f** Western blots and quantification showing indicated protein levels in tumor tissues from xenograft nude mice. **g** Diagram showing the working model. For all, $^{**}p < 0.01$; $^{***}p < 0.001$

PCED1B-AS1/hsa-mir-194-5p/PD-Ls in HCC and targeting PCED1B-AS1 might have dual effects on PD-L1 and PD-L2.

For LOC653653, LOC154761 and LINC00426, we performed knockdown of these three IncRNAs in HCC cell line and then measured the protein levels of PD-L1 and PD-L2. The results showed that knockdown of these three IncRNAs did not change PD-Ls expression (Supplementary Fig. 1D). Our interpretation is that, although their expression was correlated with PD-Ls in TCGA dataset, their expression levels were too low (TPM < 1) and they do not have biological effects on PD-Ls expression.

PD-1 antibody shows mild and insignificant benefits in HCC patients in phase III CheckMate 459 trial [24], suggesting that monotherapy against single target may not be sufficient. The same is true for PD-L1 antibody which has several clinical trials ongoing in HCC patients [25]. In such case, it would be of great interest to know whether PD-L1 and PD-L2 expression is correlated with each other. Here, we found that there was a highly positive correlation between PD-L1 and PD-L2 expression and this was cross-validated by TCGA-LIHC dataset. It suggests that HCC patients with high PD-L1 expression tend to have high PD-L2 expression and targeting PD-L1 may not show obvious benefits in these patients because PD-L2 is still functional and plays redundant roles. Thus, combinational therapies against PD-L1 and PD-L2 might further optimize the efficacy.

Insights form how PD-Ls expression is fine-tuned is essential for improving the efficacy of PD-Ls inhibitors. Previous studies have identified various proteins which regulate PD-L1 expression, such as Cdk5 [26], MYC [27] and CMTM4/CMTM6 [28]. In addition, genetic disruption of PD-L1 3′-UTR increases PD-L1 expression [29], suggesting that microRNAs also modulate PD-L1 expression. In contrast, there is no report on lncRNAs which can simultaneously modulate PD-L1 and PD-L2 so far. Here, we show that PCED1B-AS1 directly interacted with hsa-miR-194-5p which simultaneously targets PD-L1 and PD-L2. Thus, PCED1B-AS1 enhanced the expression and function of PD-L1 and PD-L2 in HCC. Meanwhile, HCC cells could release and uptake PCED1B-AS1 containing exosomes and exosomal PCED1B-AS1 could enhance PD-Ls expression in receipt HCC cells. In addition, T cells and macrophages also uptake PCED1B-AS1 containing exosomes from HCC cells and it results in inhibition of T cells and macrophages. This is fully consistent with a recent report showing that PCED1B-AS1 induces apoptosis of macrophages in Mycobacterium tuberculosis [30]. Taken together, we provide strong evidence that PCED1B-AS1 is an important regulator of PD-Ls expression and function in HCC. As in vivo knockdown of target RNA by CRISPR-CasRx has shown therapeutic potential in disease models [31], it might be feasible to target PCED1B-AS1 in near future to evaluate its therapeutic potential in HCC.

Insight from the regulatory mechanism of PD-Ls expression may also help to predict the response of patients to PD-Ls inhibitors. PD-L1 expression is predictive for the response to PD-L1 inhibitors [32] and PD-L1 expression in tumor tissues has been used as companion biomarker to identify the right patients who are most likely to respond to PD-L1 inhibitors. Thus, regulators of PD-Ls expression are also potential biomarkers to evaluate PD-Ls expression and lncRNAs may offer a unique advantage as tumor cells release exosomal lncRNAs into blood [33]. Here, we found that blood exosomal PCED1B-AS1 was highly correlated with HCC PCED1B-AS1, suggesting that HCC tissue is the major source of blood exosomal PCED1B-AS1. More importantly, blood exosomal PCED1B-AS1 was also correlated with HCC PD-L1 and PD-L2 levels, respectively. Thus, blood exosomal PCED1B-AS1 might offer a non-invasive biomarker to monitor the expression and function of PD-Ls in HCC.

Conclusions

In summary, our study suggests that PCED1B-AS1 enhances the expression and function of PD-L1 and PD-L2 via sponging miR-194-5p to induce immunosuppression in HCC. Thus, PCED1B-AS1 is a potential therapeutic target in HCC.

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Compliance with ethical standards

Conflict of interest Fei Fan, Keji Chen, Xiaoliang Lu, Aijun Li, Caifeng Liu, Bin Wu have no conflicts of interest to declare.

Ethical approval The study was approved by the Review Boards of Shanghai Eastern Hepatobiliary Surgery Hospital (Shanghai, China).

Informed consent Written informed consent was obtained from each patient.

References

1. Villanueva A. Hepatocellular carcinoma. N Engl J Med 2019;380(15):1450–1462
2. Alves RC, Alves D, Guz B, Matos C, Viana M, Harriz M, et al. Advanced hepatocellular carcinoma. Review of targeted molecular drugs. Ann Hepatol 2011;10(1):21–27
3. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. Science 2018;359(6382):1350
4. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192(7):1027–1034

5. Larsen TV, Hussmann D, Niesen AL. PD-L1 and PD-L2 expression correlated genes in non-small-cell lung cancer. Cancer Commun 2019;3:39
6. Yearley JH, Gibson C, Yu N, Moon C, Murphy E, Juco J, et al. PD-L2 expression in human tumors: relevance to anti-PD-1 therapy in cancer. Clin Cancer Res 2017;23(12):3158–3167
7. Gao Q, Wang XY, Qiu SJ, Yamato I, Sho M, Nakajima Y, et al. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. Clin Cancer Res 2009;15(3):971–979
8. Jung HI, Jeong D, Ji S, Ahn TS, Bae SH, Chin S, et al. Overexpression of PD-L1 and PD-L2 Is associated with poor prognosis in patients with hepatocellular carcinoma. Cancer Res Treat 2017;49(1):246–254
9. Yang HY, Zhou XX, Sun LJ, Mao YL. Correlation between PD-L2 expression and clinical outcome in solid cancer patients: a meta-analysis. Front Oncol 2019;13:9
10. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet 2016;17(1):47–62
11. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell 2011;147(2):358–369
12. Zhou X, Gao Q, Wang J, Zhang X, Liu K, Duan Z. Linc-RNA-RoR acts as a "sponge" against mediation of the differentiation of endometrial cancer stem cells by microRNA-145. Gynecol Oncol 2014;133(2):333–339
13. Wu XS, Wang F, Li HF, Hu YP, Jiang L, Zhang F, et al. LncRNA-PAGBC acts as a microRNA sponge and promotes gallbladder tumorigenesis. EMBO Rep 2017;18(10):1837–1853
14. Xu S, Wang Q, Kang Y, Liu J, Yin Y, Liu L, et al. Long noncoding RNAs control the modulation of immune checkpoint molecules in cancer. Cancer Immunol Res 2020;8:937–951
15. Ding J, Li X, Hu H. TarPmiR: a new approach for microRNA target site prediction. Bioinformatics 2016;32(18):2768–2775
16. Kruger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. Nucleic Acids Res 2006;34(Web server issue):W451–W454
17. Paliard X, de Waal MR, Yssel H, Blanchard D, Chretien I, Abrams J, et al. Simultaneous production of IL-2, IL-4, and IFN-gamma by activated human CD4+ and CD8+ T cell clones. J Immunol 1988;141(3):849–855
18. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol 2012;12(3):180–190
19. Yang JH, Yu D, Liu XSBJ, Changyong E, Yu S. LncRNA PCED1B-AS1 activates the proliferation and restricts the apoptosis of glioma through cooperating with miR-194-5p/PCED1B axis. J Cell Biochem 2020;121:1823–1833
20. Hou JW, Zhao RC, Xia WY, Chang CW, You Y, Hsu JM, et al. PD-L1-mediated gasdermin C expression switches apoptosis to pyroptosis in cancer cells and facilitates tumour necrosis. Nat Cell Biol 2020;22:1264–1275
21. Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. Blood 2008;111(7):3635–3643
22. Clark CA, Gupta HB, Sareddy G, Pandeswara S, Lao S, Yuan B, et al. Tumor-intrinsic PD-L1 signals regulate cell growth, pathogenesis, and autophagy in ovarian cancer and melanoma. Cancer Res 2016;76(23):6964–6974
23. Yao Z, Zhang Q, Guo F, Guo S, Yang B, Liu B, et al. Long non-coding RNA PCED1B-AS1 promotes the Warburg effect and tumorigenesis by upregulating HIF-1alpha in glioblastoma. Cell Transplant 2020;29:963689720906777
24. Yau T, Park JW, Finn RS, Cheng AL, Mathurin P, Edeline J, et al. CheckMate 459: a randomized, multi-center phase III study of nivolumab (NIVO) vs sorafenib (SOR) as first-line (1L) treatment in patients (pts) with advanced hepatocellular carcinoma (aHCC). Ann Oncol 2019;30:874
25. Kudo M. Immuno-oncology therapy for hepatocellular carcinoma: current status and ongoing trials. Liver Cancer 2019;8(4):221–238
26. Dorand RD, Nthale J, Myers JT, Barkauskas DS, Avril S, Chirieleison SM, et al. Cdk5 disruption attenuates tumor PD-L1 expression and promotes antitumor immunity. Science 2016;353(6297):399–403
27. Casey SC, Tong L, Li YL, Do R, Walz S, Fitzgerald KN, et al. MYC regulates the antitumor immune response through CD47 and PD-L1. Science 2016;352(6282):227–231
28. Mezzadra R, Sun C, Jae LT, Gomez-Eerland R, de Vries E, Wu W, et al. Identification of CMTM6 and CMTM4 as PD-L1 protein regulators. Nature 2017;549(7670):106
29. Kataoka K, Shiraishi Y, Takeda Y, Sakata S, Matsumoto M, Nagano S, et al. Aberrant PD-L1 expression through 3′-UTR disruption in multiple cancers. Nature 2016;534(7607):402
30. Li M, Cui J, Niu W, Huang J, Feng T, Sun B, et al. Long non-coding PCED1B-AS1 regulates macrophage apoptosis and autophagy by sponging miR-155 in active tuberculosis. Biochem Biophys Res Commun 2019;509(3):803–839
31. Zhou H, Su J, Hu X, Zhou C, Li H, Chen Z, et al. Glia-to-neuron conversion by CRISPR–CasRx alleviates symptoms of neurological disease in mice. Cell 2020;181(3):590 e16–603 e16
32. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 2014;515(7528):563
33. Hewson C, Morris KV. Form and function of exosome-associated long non-coding RNAs in cancer. Curr Top Microbiol 2016;394:41–56